IMMOBILIZED ChEs AND OP HYDROLASES: VERSATILE OP BIOSENSORS

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Abstract

We are developing for field use biosensor badges for organophosphates (OPs) based on immobilized enzymes coupled *in situ* with polyurethane polymer. Immobilized enzymes, cholinesterases (ChE) and/or OP hydrolases, are stable for long periods (> 3 years) and at various temperatures (to 45EC). Badges have similar kinetic parameters to soluble enzyme and selectivity to OPs as do human ChEs. Badges can be used as immediate indicators of OP contamination in diverse environments including air, water, ground, skin, or other surfaces because enzymes do not leach from the detector. OP-exposed badges are evaluated qualitatively by visual color changes or luminescence for dark-adapted eyes. The latter uses a coupled reaction of immobilized ChE, choline oxidase, and peroxidase. Badges can be used as cumulative OP dosimeters (e.g., for carbon filter capacity or pesticide exposure) when OP inhibition of ChE is irreversible. Badges can also be used to identify and quantify exposure to a specific OP. Quantitative measurements using hand-held colorimeters (UV, fluorescence, luminescence are being tested. These immobilized enzyme OP biosensor badges offer remarkable stability, sensitivity, selectivity, and rapid detection.

Introduction

Traditional analysis of cholinesterase inhibitors is performed using gas and liquid chromatography and mass spectrometry[1]. These techniques have significant drawbacks when considering an individual kit for field deployment, including lack of portability, simplicity, cost, and rapid results. An alternate technology is a biosensor, which has been widely used to detect biological, pharmacological, or clinically important compounds.

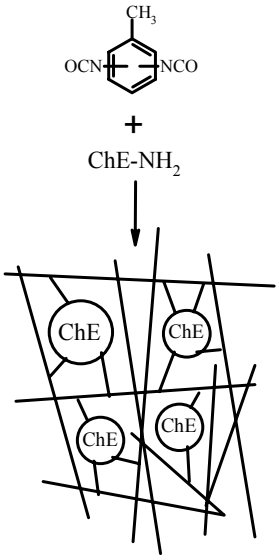
Enzyme sensors have the advantage of selectivity, sensitivity and, most important, specificity, ease and portability, and markedly simplified instrumentation. Biosensors based on cholinesterases (ChEs) immobilized *non-covalently* have been prepared by a variety of processes[2]. The drawback to these methods includes lack of enzyme stability at ambient conditions, leaching from the surface, sensitivity to denaturating conditions, and short half-life when in solution. The currently fielded spot detector, the M256A1 chemical agent detector kit, uses a dry eel ChE non-covalently applied onto a fiber. It can only be exposed to air/vapor, and there are other ChEs that provide more sensitivity, stability, specificity and usefulness under a variety of environmental conditions.

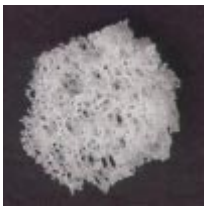
Wood and coworkers[3], using isocyanate-based polyurethane foams (Hypol), found that a number of enzymes could be covalently bound to this polymer, and that every enzyme retained activity to varying degrees. Recently, we combined a porous polyurethane foam formed *in situ* from water-miscible hydrophilic urethane prepolymers and enzymes such as ChEs, producing immobilized enzyme sponges[4,5]. In this method, the enzyme becomes a part of the solid support. Some of the advantages of this technique include resistance to denaturating buffers, and the retention of similar kinetic characteristics as the soluble form of enzyme. In addition, the enzyme will not leach from the polyurethane support so that the product - an OP badge - can now be used to sample anything from soil, water, to air. Most important, the immobilized enzyme retains most of its original activity after prolonged storage, and it is resistant to the detrimental effects of low and high temperatures.

Methods

Enzymes: AChE was purified to homogeneity from fetal bovine serum using procainamide-Sepharose 4B affinity chromatography[6], and equine serum BChE is similarly purified. Serum OP hydrolase (paraoxonase) was purified by published procedures[7]. Choline oxidase was obtained from Sigma.

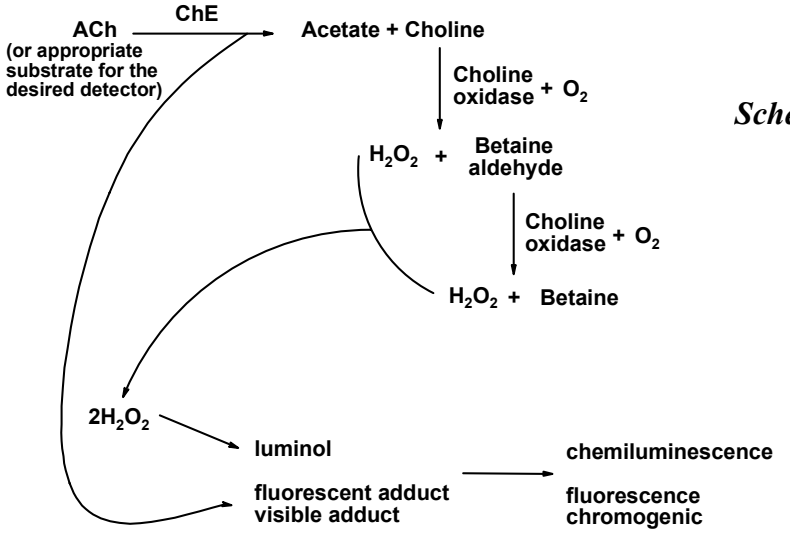
Polyurethane foam and enzyme synthesis: A typical synthesis of the sponge consists of enzyme in phosphate buffer containing 1% (final concentration) surfactant (pluronic P-65) and 6 g of water miscible prepolymer (figure, right). The functional group of Hypol prepolymer TDI 3000 is shown reacting with the surface amine group (right figure, top) of cholinesterases.[3,5]. The product is crosslinked enzyme and polurethane foam (right figure, bottom). The 2-phase system, enzyme and prepolymer, is mixed by a new method modified from the adhesive industry (figure to the left). The mixed material can be ejected into a mold to form any size





or shape product. Alternatively, the immobilized product can be spotted (as a dot of glue) onto a paper or rigid plastic backing. Note that the enzyme is not in an organic buffer as required in some immobilization techniques. The overall result is less air-induced shear producing increased activity and, importantly, simplicity. The sponge containing the covalently coupled ChEs or enzymes cures in less than 20 minutes. The PUF-sponge can be cut or bored to any desired size or shape because the enzyme(s) are homogeneously coupled throughout the sponge matrix[4]. A 5 mg sample is shown to the left (not to scale).

Determination of enzyme activity (scheme, below): Detection can be performed qualitatively by the human eye for visible chromogens, or dark-adapted eyes for chemiluminescent chromogens[8]. Detection can also be performed quantitatively using portable handheld devices, which measure fluorescence, chemiluminescence and visible chromogens.



Scheme 1

The sponges have been evaluated for activity using a modified Ellman method in an aqueous environment containing acetylthiocholine for AChE or butyrylthiocholine for BChE as substrates, respectively. The reactions were monitored spectrophotometrically at 412nm[6]. We found that there was no adsorption of the final reaction product of the Ellman assay on the polyurethane lacking ChE[4]. Additionally, product generated was linear with time, indicating release of the reaction product to the aqueous environment was not rate limiting. For OP hydrolase assay, the wavelength was 500nm and the substrate was diethyl p-nitrophenylphosphate[7]. For the choline oxidase assay, the reaction was monitored at 500nm using a coupled reaction and 4-aminoantipyrine[9].

Results

Stability: The ChE and OP hydrolase sponge is stable[4] at room temperature conditions for more than 11 months (to-date experiments), more than 3 years at 4EC, and more than 7 months at 45EC (Figure 1). The components of the badge are environmentally friendly

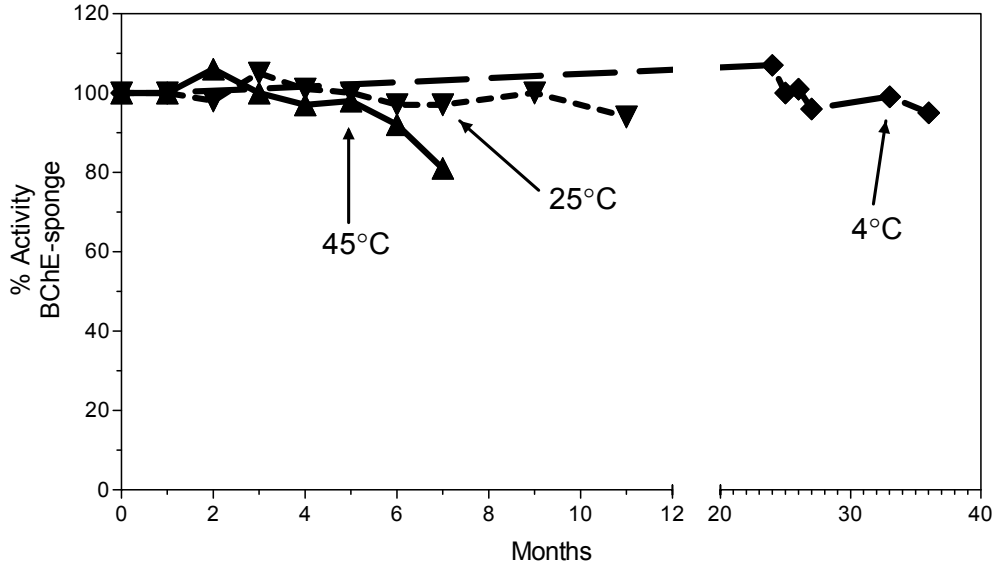


Figure 1. Stability of BChE-sponge at various temperatures. Similar results were obtained with AChE-sponge.

because the polyurethane foam, enzymes, buffers, and indicators pose no hazards. If the immobilized ChE sponge is frozen in liquid nitrogen, it can be ground to a fine powder. Most of the original activity remains, but it is unlikely that the biosensor badge would encounter these conditions in the field (-273°C). The TDI sponge imparts remarkable stability to the ChE-sponges; about 50% of the original AChE-sponge activity and 20% of the BChE-sponge activity remained after 16 hours at 80EC, conditions under which soluble enzyme would exhibit no activity. The ChE-sponges can be exhaustively dried under vacuum at 22EC and then rehydrated without loss of the enzyme activity.

Capacity of ChEs-PUF: Our results demonstrate the following characteristics of sponges containing either immobilized AChE or BChE. As expected for a uniform immobilization of AChE or BChE throughout the sponge, a linear correlation was established between the weight of the TDI sponge and enzyme activity. Sponges could be washed with either 50 mM phosphate buffer, distilled water, or 10 mM ammonium bicarbonate without affecting substrate hydrolysis. Different batches of TDI sponges retained about 50% of the original ChE activity. This demonstrates that this simple procedure of *in situ* mixing at 22EC of TDI prepolymer, surfactant, and enzyme yields a useful and effective product retaining much of the original ChE activity, while gaining remarkable stability.

We found that the TDI sponge has a significantly higher loading capacity for ChEs than the amount of purified BChE or AChE we added. As shown in figure 2, the final BChE activity of the sponge could be increased by adding larger quantities of enzyme during synthesis. When increasing amounts of nonspecific protein (bovine serum albumin, BSA) were added to a constant amount of purified AChE and the mixture cured, there was no reduction in sponge ChE activity, even when there was a 1000-fold excess

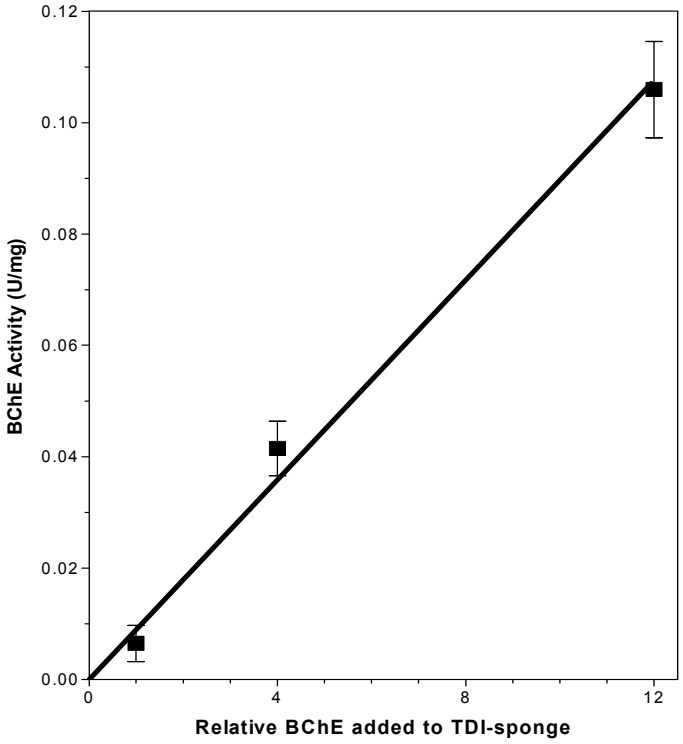


Figure 2

A linear correlation was observed between the amount of BChE added to the prepolymer during synthesis and the amount of BChE activity observed in the final sponge.

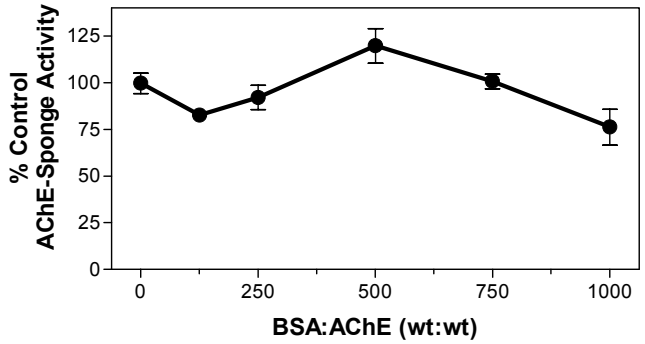


Figure 3

Increasing amounts of BSA were added during synthesis to a constant amount of AChE and TDI polymer

of BSA, as shown in figure 3. These results show that even higher potency sponges can be synthesized from purified ChEs or by synthesizing with additional enzymes and other ChEs. Therefore, sponges with combinations of multiple enzymes can be readily synthesized.

Comparison of soluble and immobilized choline oxidase: An initial rates method was used to determine the kinetic parameters for immobilized and soluble choline oxidase. As shown in figure 4 top, the plot of substrate concentration against choline oxidase activity indicates that the soluble and immobilized enzymes have similar kinetic parameters. Furthermore, the pH dependent activity of the soluble or immobilized choline oxidase are identical (figure 4 bottom). Since the pH profiles for ChEs (not shown) and choline oxidase are identical (about pH 8), the coupled reaction in the assay scheme 1 above can be simultaneous optimized for both enzymes.

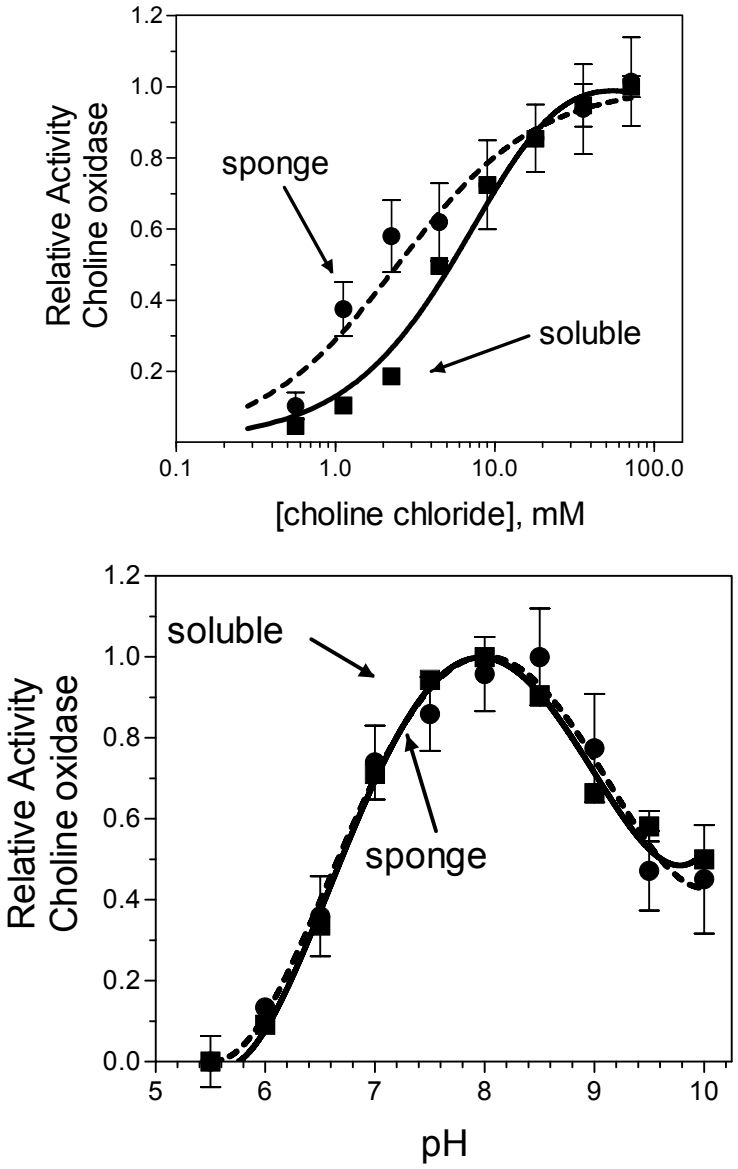


Figure 4

(top)

Substrate dependent activity of soluble and immobilized choline oxidase.

(bottom)

pH dependent activity of soluble and immobilized choline oxidase.

Conclusion

The immobilized polyurethane enzymes make versatile biosensors for detecting organophosphates. These badges, by virtue of their high capacity for enzymes, stability, sensitivity, and resistance to harsh environmental conditions, can be used under diverse conditions encountered by troops in the field. These badges should be suitable for a variety of sensor schemes for both chemical weapons and pesticides, and they could be incorporated into the telemedicine initiative as electrochemical organophosphate probes.

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